

Role of Various Enterotoxins in *Aeromonas hydrophila*-Induced Gastroenteritis: Generation of Enterotoxin Gene-Deficient Mutants and Evaluation of Their Enterotoxic Activity

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Three enterotoxins from the *Aeromonas hydrophila* diarrheal isolate SSU have been molecularly characterized in our laboratory. One of these enterotoxins is cytotoxic in nature, whereas the other two are cytotoxic enterotoxins, one of them heat labile and the other heat stable. Earlier, by developing an isogenic mutant, we demonstrated the role of a cytotoxic enterotoxin in causing systemic infection in mice. In the present study, we evaluated the role of these three enterotoxins in evoking diarrhea in a murine model by developing various combinations of enterotoxin gene-deficient mutants by marker-exchange mutagenesis. A total of six isogenic mutants were prepared in a cytotoxic enterotoxin gene (*act*)-positive or -negative background strain of *A. hydrophila*. We developed two single knockouts with truncation in either the heat-labile (*alt*) or the heat-stable (*ast*) cytotoxic enterotoxin gene; three double knockouts with truncations of genes encoding (i) *alt* and *ast*, (ii) *act* and *alt*, and (iii) *act* and *ast* genes; and a triple-knockout mutant with truncation in all three genes, *act*, *alt*, and *ast*. The identity of these isogenic mutants developed by double-crossover homologous recombination was confirmed by Southern blot analysis. Northern and Western blot analyses revealed that the expression of different enterotoxin genes in the mutants was correspondingly abrogated. We tested the biological activity of these mutants in a diet-restricted and antibiotic-treated mouse model with a ligated ileal loop assay. Our data indicated that all of these mutants had significantly reduced capacity to evoke fluid secretion compared to that of wild-type *A. hydrophila*; the triple-knockout mutant failed to induce any detectable level of fluid secretion. The biological activity of selected *A. hydrophila* mutants was restored after complementation. Taken together, we have established a role for three enterotoxins in *A. hydrophila*-induced gastroenteritis in a mouse model with the greatest contribution from the cytotoxic enterotoxin Act, followed by the Alt and Ast cytotoxic enterotoxins.

Among various *Aeromonas* species, *A. hydrophila* is most commonly involved in causing human infections such as septicemia and gastroenteritis (16). Isolation of *A. hydrophila* from water and food sources, as well as the increasing resistance of this organism to antibiotics and chlorination in water, presents a significant threat to public health (2, 4, 10, 11, 16, 26, 27, 30, 34, 48). Although *Aeromonas*-induced gastroenteritis is most common in young children, the organism is being isolated lately with high frequency from patients with traveler's diarrhea (14, 48).

The pathogenesis of *A. hydrophila* infection is complex and multifactorial, with the involvement of a number of virulence factors (1, 5). After initial colonization of the epithelial cells through type IV pili (8, 30, 31, 32), *A. hydrophila* may cause diarrhea by producing enterotoxins (14, 33). Asao et al. (6) first purified a 49- to 52-kDa β -hemolysin to homogeneity from a species of *Aeromonas* that induced fluid secretion in an animal model. Subsequently, a β -hemolysin-related aerolysin from *A. bestiarum* and *A. trota* (12, 29) and a cytotoxic enterotoxin (Act, containing 493 amino acid residues) from the *A. hydrophila* diarrheal isolate SSU were molecularly characterized (17). Act and aerolysin are pore-forming toxins, and we have demonstrated previously that Act has hemolytic, cytotoxic, and en-

terotoxic activities (17, 47). Recently, we have shown that Act activates proinflammatory cytokine and eicosanoid cascades in macrophages and a rat intestinal epithelial cell line (IEC-6), leading to tissue damage and a fluid secretory response (22; unpublished data).

In addition to Act, we have cloned two cytotoxic enterotoxin genes in *Escherichia coli* from the genomic library of *A. hydrophila* SSU (21). Unlike Act, these cytotoxic enterotoxins did not cause degeneration of crypts and villi of the small intestine (15, 20, 21). The cell lysates from *E. coli* clones harboring cytotoxic enterotoxin genes caused Chinese hamster ovary (CHO) cells to elongate and to produce cyclic AMP, which are typical enterotoxic responses (21). One of these cytotoxic enterotoxins was heat labile at 56°C and was referred to as Alt, while the other was heat stable at the same temperature and was designated Ast (20, 21). An Ast-related cytotoxic enterotoxin gene was first cloned from *A. hydrophila* (reclassified as *A. trota*) by Chakraborty et al. (13); however, it was not further characterized. These investigators demonstrated that cell lysates from their *E. coli* clone caused fluid secretion in the rabbit ligated ileal loop and suckling mouse assays (13).

We showed previously that the purified native Alt from *A. hydrophila* SSU exhibited a size of 44 kDa, elongated CHO cells, and evoked fluid secretion in the rabbit ligated ileal loop model (15, 21). More detailed characterization of the recombinant Alt from *E. coli* revealed that it consisted of a single polypeptide chain with 368 amino acid residues (20) and that purified Alt elevated cyclic AMP and prostaglandin E₂ levels in

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source ^a or reference
<i>A. hydrophila</i> SSU		CDC, Atlanta, Ga
SSU-R	Rif ^r	Laboratory stock
SSU Δ act	<i>act</i> isogenic mutant of <i>A. hydrophila</i> SSU-R generated by double crossover; Rif ^r Km ^r ; sucrose resistance	47
SSU Δ alt	<i>alt</i> isogenic mutant of <i>A. hydrophila</i> SSU-R generated by double crossover; Rif ^r Km ^r ; sucrose resistance	This study
SSU Δ ast	<i>ast</i> isogenic mutant of <i>A. hydrophila</i> SSU-R generated by double crossover; Rif ^r Km ^r ; sucrose resistance	This study
SSU Δ alt,ast	<i>alt</i> and <i>ast</i> gene double-knockout isogenic mutant of <i>A. hydrophila</i> SSU-R generated by double crossover; Rif ^r Km ^r Sm ^r Sp ^r ; sucrose resistance	This study
SSU Δ act,ast	<i>act</i> and <i>ast</i> gene double-knockout isogenic mutant of <i>A. hydrophila</i> SSU-R generated by double crossover; Rif ^r Km ^r Sm ^r Sp ^r ; sucrose resistance	This study
SSU Δ act,alt	<i>act</i> and <i>alt</i> gene double-knockout isogenic mutant of <i>A. hydrophila</i> SSU-R generated by double crossover; Rif ^r Km ^r Tc ^r ; sucrose resistance	This study
SSU Δ act,alt,ast	<i>act</i> , <i>alt</i> , and <i>ast</i> gene triple-knockout isogenic mutant of <i>A. hydrophila</i> SSU-R generated by double crossover; Rif ^r Km ^r Sm ^r Sp ^r Tc ^r ; sucrose resistance	This study
<i>E. coli</i>		
HB101	<i>recA13 hsdS20 supE44</i>	Promega
DH5 α	<i>recA gyrA</i>	Laboratory stock
SM10	Km ^r λ pir	35
S17-1	Sm ^r ; trimethoprim resistance; λ pir	28
HMS174(DE3)	Rif ^r	Novagen
Plasmids		
pRK2013	Helper plasmid, Km	ATCC, Manassas, Va.
pBR322	Ap Tc	Amersham
pBluescript-SK	Ap	Stratagene
pUC-4K	Contains a 1.2-kb Km gene cassette	Amersham
pHP45 Ω	Contains a 2.0-kb Sm/Sp gene cassette	40
pJQ200SK	Suicide vector; P15A <i>ori sacB</i> Gm	28, 41
pDMS197	Suicide vector; R6K <i>ori sacB</i> Tc	24
pRE112	Suicide vector; R6K <i>ori sacB</i> Cm	24
pSL24	Contains a 4.0-kb <i>SalI</i> DNA fragment harboring the <i>alt</i> gene in pT7-6 vector; Ap	21
pSBS32	Contains a 6.0-kb <i>SalI</i> DNA fragment harboring the <i>ast</i> gene in pBluescript-SK vector; Ap	21
pBlue <i>alt</i>	pBluescript recombinant plasmid with a 4.0-kb <i>SalI</i> DNA fragment from plasmid pSL24 containing the <i>alt</i> gene	This study
pBlue <i>ast</i>	pBluescript recombinant plasmid with a 4.6-kb <i>SalI/BamHI</i> DNA fragment from plasmid pSBS32 containing the <i>ast</i> gene	This study
pB <i>ast</i> -Sm/Sp	<i>ast</i> gene in plasmid pBlue <i>ast</i> truncated at the <i>SmaI</i> site with a Sm-Sp gene cassette	This study
pB <i>ast</i> -Km	<i>ast</i> gene in plasmid pBlue <i>ast</i> truncated at the <i>SmaI</i> site with a Km gene cassette	This study
pB <i>alt</i> -Km	<i>alt</i> gene in plasmid pBlue <i>alt</i> truncated at the <i>BglII</i> site with a Km gene cassette	This study
pB <i>alt</i> -Tc	<i>alt</i> gene in plasmid pBlue <i>alt</i> truncated at the <i>BglII</i> site with a Tc gene cassette	This study
pJQ <i>alt</i> -Km	Vector pJQ200SK containing a Km gene cassette-truncated <i>alt</i> gene with its flanking sequences for generating mutant SSU Δ alt	This study
pJQ <i>ast</i> -Km	Vector pJQ200SK containing a Km gene cassette-truncated <i>ast</i> gene with its flanking sequences for generating mutant SSU Δ ast	This study
pDMS <i>ast</i> -Sm/Sp	Vector pDMS197 containing a Sm-Sp gene cassette-truncated <i>ast</i> gene with its flanking sequences for generating SSU Δ alt,ast and SSU Δ act,ast mutants	This study
pRE <i>alt</i> -Tc	Vector pER112 containing a Tc gene cassette-truncated <i>alt</i> gene with its flanking sequences for generating SSU Δ act,alt and SSU Δ act,alt,ast mutants	This study
pBR <i>alt</i>	<i>A. hydrophila alt</i> gene with its putative promoter region, cloned in pBR322 at the <i>EcoRI</i> site	This study
pBR <i>ast</i>	<i>A. hydrophila ast</i> gene with its putative promoter region, cloned in pBR322 at the <i>EcoRI/PstI</i> site	This study
pBR <i>act</i>	<i>A. hydrophila act</i> gene with its putative promoter region, cloned in pBR322 at the <i>EcoRI/PstI</i> site	This study
pGPI-2	Contains a T7 RNA polymerase gene which is regulated by a thermally inactivated repressor, <i>cI857</i> ; Km	Laboratory stock
pT7-5	Contains a T7 promoter upstream of the multiple cloning site; Ap	Laboratory stock
pT7-6	Contains a T7 promoter upstream of the multiple cloning site; the multiple cloning site is in the opposite orientation from the pT7-5 vector; Ap	Laboratory stock

^a CDC, Centers for Disease Control and Prevention; ATCC, American Type Culture Collection.

CHO and rat intestinal epithelial cells (20, 21). Our recent DNA sequence analysis of the *ast* gene, which is presented in this paper, revealed that it was encoded by a 1,911-bp open reading frame (ORF), contained 636 amino acid residues, and had a predicted molecular mass of 71 kDa with an isoelectric point of 6.9 based on computer analysis. Both Alt and Ast represent novel molecules with no significant homology to known bacterial enterotoxins (15, 20, 21).

The molecular characterization of these three enterotoxins (Act, Alt, and Ast) (15, 17, 20, 21) will now allow us to define their individual contributions in evoking diarrhea and a possible interaction among these enterotoxins during *A. hydrophila* infections. In this report, we have generated various enterotoxin gene-deficient mutants of *A. hydrophila* to precisely evaluate their role in secretory diarrhea.

MATERIALS AND METHODS

Bacterial strains and plasmids. The sources of *A. hydrophila* and *E. coli* strains, as well as the plasmids used in this study, are listed in Table 1. Briefly, the pBlue *alt* recombinant plasmid contained a 4.0-kb *SalI* DNA fragment from the chromosome of *A. hydrophila* SSU that harbored the *alt* gene in pBluescript (Stratagene, La Jolla, Calif.) and was generated from the original pSL24 recombinant plasmid (21). The pBlue *ast* recombinant plasmid contained a 4.8-kb *SalI/BamHI* *A. hydrophila* SSU chromosomal DNA fragment in pBluescript and harbored the *ast* gene (21). This plasmid was generated from the original clone pSBS32, which contained a 6.0-kb *SalI* DNA fragment harboring the *ast* gene (21). The suicide vector pJQ200SK contained a P15A origin of replication (*ori*), a levansucrase gene (*sacB*) from *Bacillus subtilis*, and a gentamicin resistance (Gm) gene (28, 41). Suicide vectors pDMS197 and pRE112 had a conditional R6K *ori* and a *sacB* gene and also a tetracycline resistance (Tc) gene and a chloramphenicol resistance (Cm) gene, respectively (24). An *act* isogenic mutant of *A. hydrophila* SSU (designated SSU Δ act) was previously generated in our laboratory via homologous recombination using suicide vector pJQ200SK (47).

Enzymes, chemicals, and recombinant DNA techniques. The antibiotics ampicillin, gentamicin (GEN), tetracycline (TET), kanamycin (KAN), chloramphenicol, spectinomycin (SPT), and streptomycin (STR) were used at concentrations of 100, 15, 15, 50, 20, 25, and 50 µg/ml, respectively, unless otherwise stated. Rifampin (RIF) was used at a concentration of 40 µg/ml for bacterial growth and 300 µg/ml during conjugation experiments. All of the antibiotics used were obtained from Sigma (St. Louis, Mo.). Restriction endonucleases and T4 DNA ligase were obtained from Promega (Madison, Wis.) and New England BioLabs (Beverly, Mass.). The Advantage cDNA PCR kit was purchased from Clontech (Palo Alto, Calif.). Chromosomal DNA from various *A. hydrophila* mutants was isolated with a QIAamp DNA Mini kit (Qiagen, Inc., Valencia, Calif.). The plasmid DNA and the DNA fragments from the agarose gel were prepared and purified with a QIAprep Miniprep Kit (Qiagen). All of the basic molecular biology techniques used in this study were previously described (7, 47).

Expression of the *ast* gene with a bacteriophage T7 promoter-polymerase system. A dual-plasmid T7 expression system developed by Tabor and Richardson (46) was used for the expression of the *ast* gene. The recipient *E. coli* strain HB101 contained a plasmid, pGP1-2, with a T7 RNA polymerase gene whose expression was regulated by a thermally inactivated repressor, *cI857*, and also a Km gene. The 4.8-kb *SalI/BamHI* DNA fragment containing the *ast* gene was cloned in Ap plasmid vectors pT7-5 and pT7-6, which had multiple cloning sites in opposite orientations and a promoter for the T7 RNA polymerase gene, located upstream of the multiple cloning sites. The recombinant pT7-5 and pT7-6 plasmids were transformed into *E. coli* HB101(pGP1-2) (39) and examined for the expression of the *ast* gene. Briefly, 2 ml of the recombinant *E. coli* clone harboring the *ast* gene was grown in Luria-Bertani (LB) medium (39, 42) at 30°C with 100 µg of ampicillin/ml and 40 µg of KAN/ml to an optical density at 600 nm (OD₆₀₀) of 0.5. The cells were collected by centrifugation and washed three times with M9 medium (39, 42). The pellet was resuspended in 1 ml of M9 medium supplemented with 20 µg of thiamine/ml and 0.01% 18-amino-acid mixture (minus methionine and cysteine) and grown at 30°C for 60 min with shaking (180 rpm). To induce the gene for T7 RNA polymerase, the growth temperature of the culture was shifted to 42°C for 15 min and RIF (200 µg/ml) was added for an additional 10 min to inhibit *E. coli* endogenous RNA polymerase activity, followed by incubation at 30°C for 20 min. Newly synthesized proteins were labeled by adding 30 µCi of [³⁵S]methionine-cysteine (ICN, Irvine, Calif.) during the last 5 min of incubation of the culture at 30°C. The labeled proteins were precipitated with 10% trichloroacetic acid on ice for 30 min. After centrifugation, the pellet was washed three times with cold 10% trichloroacetic acid and dissolved in 100 µl of the sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer (42). Protein samples were subjected to electrophoresis and autoradiography (39). For preparing cell lysate from an *E. coli* clone expressing the *ast* gene with the T7 expression system for biological activity measurement, 100 ml of LB medium was inoculated with the culture and the culture was grown at 30°C and shaken with appropriate antibiotics until an OD₆₀₀ of 1.0 was reached. The culture was induced at 42°C for 25 min, and then 400 µg of RIF/ml was added. The temperature of the culture was reduced to 37°C for an additional 2 h, the cells were harvested and sonicated, and the cell lysate was examined for biological activity (15, 47).

DNA sequencing of the *ast* gene. The entire 4.8-kb *SalI/BamHI* DNA fragment containing the *ast* gene was sequenced using a 373XL automated DNA sequencer (Applied Biosystems, Inc., Foster City, Calif.) in the Protein Chemistry Core Laboratory, University of Texas Medical Branch. The new primers were designed based on the confirmed sequence of the *ast* gene, and both strands of the DNA were sequenced.

Development of single-knockout mutants of *A. hydrophila* SSU with truncation in either the *alt* or the *ast* gene. As shown in Fig. 1, the plasmid pBlue *alt* containing a 4.0-kb *SalI* DNA fragment with the *alt* gene from the chromosomal DNA of *A. hydrophila* was used to prepare the *alt* isogenic mutant (SSUΔ*alt*). In the *alt* gene, there was a unique *BglII* restriction site; the plasmid pBlue *alt* was thus linearized with *BglII* enzyme (Fig. 1). A 1.2-kb Km gene cassette was isolated from plasmid pUC4K (Amersham Pharmacia Biotech, Piscataway, N.J.) by using restriction enzyme *BamHI*, which bordered the Km gene cassette. This Km gene cassette was ligated to plasmid pBlue *alt* at the *BamHI*-compatible *BglII* restriction site to truncate the *alt* gene, which generated a new recombinant plasmid, pB *alt*-Km (Fig. 1). Subsequently, the *SalI* DNA fragment, which was now 5.2 kb in size due to the insertion of a 1.2-kb Km gene cassette, was removed from the plasmid pB *alt*-Km by *SalI* digestion and ligated to a suicide vector, pJQ200SK, at the *SalI* site, forming a new recombinant plasmid, pJQ *alt*-Km, in *E. coli* strain S17-1 (Fig. 1 and Table 1). This strategy to prepare an isogenic mutant provided 1.2 and 2.8 kb of the 5' and 3' DNA sequences flanking the truncated *alt* gene, respectively, to permit double-crossover homologous recombination.

To generate an SSUΔ*ast* mutant of *A. hydrophila*, the *ast* gene was cleaved at the unique *SmaI* restriction site within the 4.8-kb *SalI/BamHI* DNA fragment in plasmid pBlue *ast* (Fig. 1 and Table 1). Subsequently, the *ast* gene was truncated with the Km gene cassette, which was removed from the plasmid pUC4K by *PstI* digestion. The *PstI* restriction sites bordered the Km gene cassette. The ends of the Km gene cassette were made blunt with a PCR polishing kit (Stratagene), and the cassette was ligated at the blunt *SmaI* restriction site within the *ast* gene to create the pB *ast*-Km plasmid (Fig. 1). The truncated *ast* gene with its flanking sequences was removed by *SalI/BamHI* digestion of the plasmid pB *ast*-Km and ligated to the suicide vector pJQ200SK at the compatible restriction sites, forming a recombinant plasmid, pJQ *ast*-Km, in *E. coli* strain S17-1 (Fig. 1). This strategy provided 2.5 and 2.1 kb of the 5' and 3' DNA sequences flanking the truncated *ast* gene, respectively, to allow double-crossover homologous recombination.

The recombinant *E. coli* S17-1(pJQ *alt*-Km or pJQ *ast*-Km) strain (Fig. 1) was conjugated with Rif^r *A. hydrophila*, as described previously for the development of an *act* isogenic mutant (SSUΔ*act*) (47). The transconjugants were plated onto LB agar plates with RIF, KAN, and 5% sucrose to select double-crossover transconjugants (47). The cultures were identified as *Aeromonas* by a positive oxidase test to differentiate them from *E. coli* and by an automated identification system (Vitek, Hazelwood, Mo.) (47).

Construction of recombinant plasmids pDMS *ast*-Sm/Sp and pRE *alt*-Tc for developing double- and triple-knockout mutants of *A. hydrophila* SSU. The pBlue *ast* plasmid, as described above, was linearized with *SmaI* restriction enzyme (Fig. 1). Subsequently, a 2.0-kb Sm-Sp gene cassette was isolated from plasmid pH45Ω by digestion with *SmaI* enzyme, which bordered the Sm-Sp gene cassette, and the 2.0-kb Sm-Sp gene cassette then was inserted at the *SmaI* restriction site of the *ast* gene to create the pB *ast*-Sm/Sp plasmid (Fig. 1). Finally, a 5-kb *KpnI/XbaI* DNA fragment from this plasmid, containing the *ast* gene with a 2.0-kb Sm-Sp gene cassette, was cloned at the *KpnI/XbaI* restriction sites of a pDMS197 suicide vector containing a Tc gene to create a recombinant plasmid, pDMS *ast*-Sm/Sp (Fig. 1).

Likewise, the pBlue *alt* plasmid, as described previously, was linearized with the *BglII* restriction enzyme, which cleaved the *alt* gene, and truncated with a 1.3-kb Tc gene cassette obtained by PCR amplification from plasmid pBR322 by using specific primers (Fig. 1; Table 2). A 5.3-kb *KpnI/XbaI* DNA fragment containing the *alt* gene with the Tc gene cassette from plasmid pB *alt*-Tc was subcloned in the suicide vector pRE112 containing the Cm gene, to generate a recombinant plasmid, pRE *alt*-Tc (Fig. 1). The recombinant plasmids pDMS *ast*-Sm/Sp and pRE *alt*-Tc were transformed into *E. coli* SM10, as described previously (45, 47). Both *E. coli* strains, S17-1 and SM10, contained *λpir*, allowing replication of the suicide vectors only in these strains (24, 28).

Construction of double-knockout mutants of *A. hydrophila* SSU. To generate an *alt*- and *ast*-negative mutant of *A. hydrophila* (SSUΔ*alt,ast*), *E. coli* SM10 *λpir*(pDMS *ast*-Sm/Sp) (Fig. 1) and the Rif^r and Km^r SSUΔ*alt* mutant, as developed above, were used for conjugation. Cultures that were resistant to KAN, STR-SPT, RIF, and 5% sucrose should have represented genuine double-crossover mutants. For developing an *act*- and *ast*-negative mutant of *A. hydrophila* (SSUΔ*act,ast*), *E. coli* SM10 *λpir*(pDMS *ast*-Sm/Sp) (Fig. 1) and the Rif^r and Km^r SSUΔ*act* mutant, as previously developed (47), were used for conjugation. Cultures resistant to KAN, STR-SPT, RIF, and 5% sucrose were analyzed. For developing an *act*- and *alt*-negative mutant of *A. hydrophila* (SSUΔ*act,alt*), *E. coli* SM10 *λpir*(pRE *alt*-Tc) (Fig. 1) and the Rif^r and Km^r SSUΔ*act* mutant, as previously developed (47), were used for conjugation. Cultures resistant to KAN, TET, RIF, and 5% sucrose should have represented double-crossover mutants.

Construction of a triple-knockout mutant of *A. hydrophila* SSU. To generate an *act*-, *alt*-, and *ast*-negative mutant of *A. hydrophila* (SSUΔ*act,alt,ast*), a double-knockout mutant (SSUΔ*act,ast*), as developed above, and *E. coli* SM10 *λpir*(pRE *alt*-Tc) (Fig. 1) were used for conjugation. Cultures resistant to KAN, STR-SPT, TET, RIF, and 5% sucrose should have represented genuine triple-knockout mutants. The identity of various mutants developed was confirmed by PCR, Southern blot analysis, and DNA sequence analysis.

Southern blot analysis. The chromosomal DNA from different enterotoxin gene-deficient mutants, as well as the wild-type *A. hydrophila*, was isolated, and an aliquot (10 µg) of the chromosomal DNA was digested with appropriate restriction enzymes and subjected to 0.8% agarose gel electrophoresis (42, 47). Next, the digested DNA was transferred to a nylon membrane (Gibco BRL, Gaithersburg, Md.) and baked at 80°C for 2 h. The blots were prehybridized and hybridized by using Quikhyb (Stratagene) at 68°C, as described by the manufacturer. The blots were hybridized using *alt* and *ast* gene probes. The full-length toxin genes for hybridization were obtained by PCR amplification from the chromosomal DNA of *A. hydrophila* SSU. The sequences of the primers used for PCR amplification of the toxin genes are shown in Table 2. The primers were

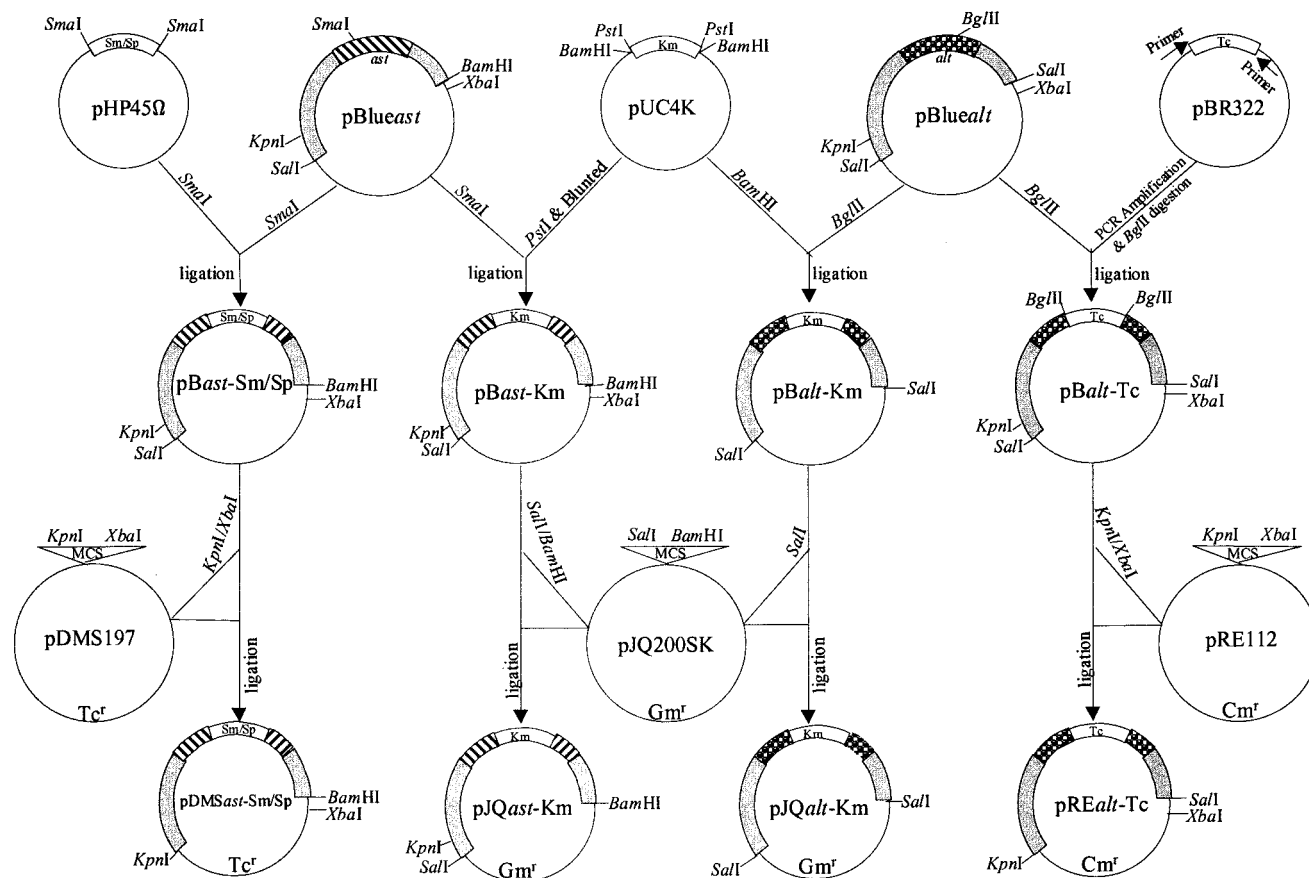


FIG. 1. Flow diagram showing the construction of various recombinant plasmids for the preparation of enterotoxin gene-deficient mutants of *A. hydrophila* SSU. The recombinant plasmid pBlue_{ast} contained a 4.6-kb *Sal*I/*Bam*HI DNA fragment from the chromosome of *A. hydrophila* which harbored the *ast* gene. The *ast* gene was truncated at the *Sma*I restriction site by introducing either a *Km* gene cassette from plasmid pUC4K or a *Sm*-*Sp* gene cassette from plasmid pHP45Ω to generate recombinant plasmid pB_{ast}-*Km* or pB_{ast}-*Sm/Sp*, respectively. The truncated *ast* gene with its flanking sequences was cloned into suicide vector pJQ200SK or pDMS197, forming recombinant plasmid pJQ_{ast}-*Km* or pDMS_{ast}-*Sm/Sp*, respectively, for the generation of *ast* gene-deficient mutants of *A. hydrophila*. The recombinant plasmid pBlue_{alt} contained a 4.0-kb DNA fragment from the chromosome of *A. hydrophila* which harbored the *alt* gene. The *alt* locus was truncated at the *Bgl*II restriction site by introducing either a *Km* gene cassette from plasmid pUC4K or a *Tc* gene cassette from plasmid pBR322 to generate recombinant plasmid pB_{alt}-*Km* or pB_{alt}-*Tc*, respectively. The truncated *alt* locus with its flanking sequences was cloned into suicide vector pJQ200SK or pRE112, forming recombinant plasmid pJQ_{alt}-*Km* or pRE_{alt}-*Tc*, respectively, for the generation of *alt* gene-deficient mutants of *A. hydrophila*. The striped bar represents the *ast* gene, while the dotted bar represents the *alt* gene. The gray bar represents sequences flanking the *ast* or *alt* gene. The open bar indicates the *Km*, *Sm*-*Sp*, or *Tc* gene cassette. These plasmids are not drawn to scale. MCS, multiple cloning sites. The primers used to amplify the *Tc* gene cassette had a *Bgl*II restriction site (Table 2).

synthesized commercially by Biosynthesis, Inc. (Lewisville, Tex.), and the program used for PCR was as follows: 94°C for 2 min (denaturation), followed by 30 cycles of 94°C for 1 min and 68°C for 3 min. The final extension was performed at 72°C for 7 min. The PCR-amplified products were isolated from the agarose gel and purified by using a QIAquick Gel Extraction kit (Qiagen). Similarly used probes for Southern blot analysis were the *Km* gene cassette (1.2 kb) obtained from the plasmid pUC4K by *Pst*I restriction enzyme digestion, the *Sm*-*Sp* gene cassette (2.0 kb) recovered from plasmid pHP45Ω by *Sma*I restriction enzyme digestion, and the *Tc* gene cassette (1.3 kb) obtained by PCR amplification from the plasmid pBR322 using specific primers (Table 2). The suicide vector plasmids (pJQ200SK, pDMS197, and pRE112) were probed against linearized plasmids after digestion with restriction enzyme *Xba*I. The probes were labeled with [α -³²P]dCTP (ICN) by using a random primer kit (Gibco BRL). The membranes were washed twice at 68°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0) (42, 47) plus 0.1% SDS for 20 min and then twice in 1× SSC plus 0.1% SDS for 20 min at 68°C. The blots were exposed to the X-ray film at -70°C for 2 to 12 h.

Northern blot analysis. Wild-type *A. hydrophila* SSU and its various enterotoxin gene-deficient isogenic mutants were grown in LB medium at 37°C overnight. The next morning, 200 μl of the overnight cultures was added to 4 ml of the fresh LB medium in 50-ml sterilized disposable tubes and allowed to grow for

another 3 h. The cells were collected by centrifugation, and the total RNA was isolated using the RNA isolation kit from Qiagen. The RNA samples (10 μg) were subjected to electrophoresis on a 1.2% formaldehyde-agarose gel with 1× MOPS buffer (0.2 M MOPS [morpholinepropanesulfonic acid] [pH 7.0], 0.005 M sodium acetate, 0.01 M EDTA, pH 8.0) (42, 47). The RNA was transferred to the nylon membrane, and after baking, the filters were prehybridized, hybridized, and washed as described for Southern blot analysis. The ³²P-labeled various gene probes were used for hybridization. The amount of RNA in each lane was quantitated by scanning 23S or 16S rRNA bands after ethidium bromide staining of the gel, using the Gel Doc 2000 System (Bio-Rad Laboratories, Hercules, Calif.). All of the reagents used for Northern blot analysis were treated with diethyl pyrocarbonate (Sigma).

Purification of Ast and Western blot analysis. The coding region of the *ast* gene (1,911 bp) generated by PCR amplification from the pBlue_{ast} plasmid with specific primers with 5' *Nde*I and 3' *Xho*I restriction sites (Table 2) was ligated to a bacteriophage T7 polymerase-promoter-based pET30a vector (Novagen, Madison, Wis.) at the *Nde*I/*Xho*I sites and transformed into *E. coli* HMS174(DE3). This vector had six histidine residues, which were fused in frame with the protein of interest at the C-terminal end for affinity purification on a nickel column. The recombinant *E. coli* clone (pT30_{ast}) was grown in LB medium (200 ml) with shaking (180 rpm) to an OD₆₀₀ of 0.6 before induction

TABLE 2. Sequences of the primers used for amplification of various toxin genes and the antibiotic resistance cassettes

Primer position	Primer sequence ^a	Location	Reference	Purpose
5'	5' CGTGGATCCATGCAAAATAAATAACTGGC 3' with <i>Bam</i> HI site	+1 to +24 of the <i>act</i> gene	17	Amplification of the <i>act</i> gene for probe preparation
3'	5' TTATTGATGGCTGGTGCACGCT 3'	+1456 to +1482 of the <i>act</i> gene	17	
5'	5' ATAGAGGAATTCCTCCATGATCGCCGGGCTGTGGGGCGGGCGG 3'	+72 to +103 of the <i>alt</i> gene	20	Amplification of the <i>alt</i> gene for probe preparation and identification of the <i>alt</i> isogenic mutant by PCR
3'	5' CATCCTAAGCTTTAAGCTTTCAACGCGCCATCGCAACGCTCTC 3' with <i>Eco</i> RI (5' primer) and <i>Hind</i> III (3' primer) sites	+1082 to +1114 of the <i>alt</i> gene	20	
5'	5' ATGCACGCACGTACCGCCATG 3' ^b	+1 to +21 of the <i>ast</i> gene	This study	Amplification of the <i>ast</i> gene for probe preparation and identification of the <i>ast</i> isogenic mutant by PCR
3'	5' GGACTTTTCACCGCAGCGGGTT 3' ^b	+1886 to +1908 of the <i>ast</i> gene	This study	
5'	5' TAGAGATCTGAATTCATGTTGACAGC 3' with <i>Bgl</i> II site	-3 to +17 of plasmid pBR322	42	Amplification of the Tc cassette for the truncation of the <i>alt</i> gene and probe preparation
3'	5' GCTAGATCTCAAGGGTTGGTTGGGCAT 3' with <i>Bgl</i> II site	+1355 to +1374 of plasmid pBR322	42	
5'	5' CTGAATTCACATGATCGTGGCCACCGACT 3' with <i>Eco</i> RI site	-543 to -523 of the <i>alt</i> gene	Unpublished data	Amplification of the <i>alt</i> gene and its putative promoter region for complementation
3'	5' CTGAATTCACCGCGCCATCGCCACGATCT 3' with <i>Eco</i> RI site	+1084 to +1107 of the <i>alt</i> gene	20	
5'	5' AACTGCAGATGACCTCATAGTAGAC 3' with <i>Pst</i> I site	-532 to -514 of the <i>ast</i> gene	This study	Amplification of the <i>ast</i> gene and its putative promoter region for complementation
3'	5' AAGAAATTCAGGACTTTTTCACCGCAGC 3' with <i>Eco</i> RI site	+1891 to +1911 of the <i>ast</i> gene	This study	
5'	5' CGCTAGGCTCTGCCCTCGTGAAGAAGGTGTT 3'	+434 to +464 of the pUC4K plasmid	18	Amplification of the Km gene cassette for PCR identification of the generated mutants
3'	5' AAAGCCACGTTGTCTAAATCTCTGATGT 3'	+1613 to +1643 of the pUC4K plasmid	18	
5'	5' CTATGGCGCATCAACAGCTCGCC 3'	+423 to +446 of the <i>ast</i> gene	This study	Sequencing primers for further identification of the truncated <i>ast</i> gene in the mutants
3'	5' GCGATAGCTGAGCGGCTTGCCCTG 3'	+553 to +576 of the <i>ast</i> gene	This study	
5'	5' CTCAACACCATCACCGACGTG 3'	+307 to +327 of the <i>alt</i> gene	20	Sequencing primers for further identification of the truncated <i>alt</i> gene in the mutants
3'	5' GCTCAGGGCGAAGCCGGCTC 3'	+454 to +474 of the <i>alt</i> gene	20	
5'	5' GTCTGCAGATGGCCGACGCCATCG 3' with <i>Pst</i> I site	-546 to -529 of the <i>act</i> gene	This study	Amplification of the <i>act</i> gene and its putative promoter region for complementation
3'	5' TGGAAATCTATTGATGGCTGCTGGCGT 3' with <i>Eco</i> RI site	+2345 to +2365 of the <i>act</i> gene	This study	

^a Underlining indicates restriction enzyme sites in the primer.

^b Similar primers with *Nde*I (5' primer) and *Xho*I (3' primer) restriction enzyme sites were used for amplifying the coding region of the *ast* gene and cloning into the pET30a vector.

with 1 mM isopropylthio- β -galactoside (IPTG) for 4 h at 37°C. The expression of the *ast* gene was monitored by SDS-12% PAGE and Coomassie blue staining, with uninduced culture as a control. For purification of Ast, the cells were sonicated after IPTG induction and the pellet was solubilized in 8 M urea in denaturing binding buffer (Invitrogen, Carlsbad, Calif.). The sample was loaded onto a nickel column (Invitrogen), and Ast was eluted with 150 to 200 mM imidazole. The identity of Ast was confirmed by microsequencing of Ast from the Immobilion-P membrane after SDS-PAGE (19) with a 494HT microsequencer (Applied Biosystems, Inc.) at the Protein Chemistry Core Laboratory, University of Texas Medical Branch.

The antibodies to Ast were generated in mice after intraperitoneal injection with polyacrylamide gel pieces containing Ast mixed with Freund's incomplete adjuvant. The animals were bled before immunization and every 2 weeks after immunization, with booster antigen given every week for 2 months. The antibody titer was determined by an enzyme-linked immunosorbent assay with Ast as the source of antigen (15). To examine expression of the *ast* gene in various enterotoxin gene-deficient mutants of *A. hydrophila*, the cultures were grown to an OD₆₀₀ of 0.6. A 2-ml suspension of the culture was centrifuged, and the pellet was dissolved in 200 μ l of the SDS sample buffer (42). An aliquot (5 μ l) of each sample was subjected to SDS-12% PAGE and Western blot analysis (17). Specific polyclonal antibodies to Ast were used as primary antibodies for Western blot analysis, followed by appropriate secondary antibodies, which were labeled with horseradish peroxidase (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.). The blots were developed by using SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, Ill.).

Complementation of SSU Δ alt, SSU Δ ast, and SSU Δ act mutants of *A. hydrophila* SSU. By using specific primers (Table 2) with an *Eco*RI restriction site, a 1.5-kb DNA fragment containing the *alt* gene and its putative promoter region was amplified from the chromosomal DNA of *A. hydrophila*. It was then ligated to the vector pBR322 at the *Eco*RI restriction site to generate recombinant plasmid pBR *alt* (Table 1), which was first transformed into *E. coli* HB101, which carried a helper plasmid, pRK2013 (with the Km gene). Subsequently, via conjugation, the recombinant pBR *alt* plasmid with the helper plasmid pRK2013 was transformed into an *alt* gene-deficient mutant of *A. hydrophila* (SSU Δ alt) (Table 1) that was generated during this study by double-crossover homologous recombination (Fig. 1). The transconjugants were screened on LB agar plates containing RIF, KAN, and TET. The presence of the pBR *alt* recombinant plasmid in the *A. hydrophila* SSU Δ alt mutant was confirmed by plasmid isolation and restriction enzyme analysis.

By using a strategy similar to that described above and specific primers (Table 2), we amplified a 2.5-kb DNA fragment containing the *ast* gene and its putative promoter region from *A. hydrophila* chromosomal DNA. This DNA fragment was ligated to the vector pBR322 at the *Eco*RI/*Pst*I restriction sites to generate the recombinant plasmid pBR *ast* (Table 1). Subsequently, the pBR *ast* plasmid was transformed into an *ast* gene-deficient mutant (SSU Δ ast), as prepared for the experiment described for Fig. 1.

For SSU Δ act complementation, a pair of primers was designed (Table 2) to amplify a 2.0-kb DNA fragment containing the *act* gene and its putative promoter region from *A. hydrophila* chromosomal DNA. The amplified DNA fragment was subsequently inserted into vector pBR322 at *Eco*RI/*Pst*I restriction sites to generate recombinant plasmid pBR *act* in *E. coli* HB101 with helper plasmid pRK2013 (Table 1). The pBR *act* plasmid was then transformed from *E. coli* into SSU Δ act by conjugation as previously described. The wild-type *A. hydrophila* transformed with pBR322 vector alone served as a control.

CHO cell elongation assay. The assay was performed as previously described (38). Briefly, the 96-well culture plates were seeded with 10⁴ CHO cells/200 μ l in F-12 medium, which contained penicillin (100 U/ml), STR (100 μ g/ml), GEN (50 μ g/ml), and fetal bovine serum (1%). The CHO cells were incubated at 37°C with 5% CO₂ for 1 h. Subsequently, a twofold dilution of cell lysates containing Ast was added to the cells, and the plates were incubated for 24 h at 37°C. The cells were fixed with 70% methanol, stained with Giemsa stain, and examined for CHO cell elongation.

Hemolytic assay. A twofold dilution of the culture filtrates from wild-type *A. hydrophila* and its various isogenic mutants was prepared in phosphate-buffered saline (PBS) and mixed with rabbit red blood cells (2%; Colorado Serum Co., Denver, Colo.) in a microtiter plate (47). The plate was incubated at 37°C for 1 h and subsequently at 4°C overnight. The hemolytic activity unit was defined as the reciprocal dilution of Act in the culture filtrate demonstrating 50% lysis of red blood cells. The culture filtrates were prepared by growing cultures in LB medium for 18 h at 37°C. After centrifugation, the culture filtrates were filter sterilized before measuring hemolytic activity (17).

Mouse and rat ligated ileal loop assay. A diet-restricted, antibiotic-treated adult mouse model was used to evaluate the enterotoxic activity of various

enterotoxin gene-deficient mutants of *A. hydrophila* SSU. Briefly, 20- to 25-g BALB/c mice (Taconic Farms, Inc., Germantown, N.Y.) were restricted in food intake by 20% for 3 weeks and then given STR (5 g/liter in drinking water) for 48 h prior to intraluminal challenge with the organism. The antibiotic was removed from water 6 h prior to surgery. The mice were anesthetized with halothane (Halocarbon Laboratories, River Edge, N.J.). An abdominal incision was made, and a single 5-cm segment of the small intestine was constructed with 00-size silk suture (18). Approximately 2 \times 10⁵ CFU (per 100 μ l) of the test organism grown in LB medium was inoculated in the ligated small intestinal loop of each mouse (18). After 12 to 16 h of observation, the animals were euthanized by cervical dislocation, the intestinal loops were excised, and the fluid was collected in a microcentrifuge tube. The microcentrifuge tubes were centrifuged briefly, and the amount of luminal fluid was measured (18). When rats were used, the animals (70 to 75 g; Sprague-Dawley, Indianapolis, Ind.) were anesthetized with 35 mg of pentobarbital sodium (Nembutal)/kg of body weight intraperitoneally. After an abdominal incision, the rat intestine was ligated into 5-cm segments, as described above, and injected with 0.5 ml of the cell lysates from *E. coli* clones containing the *ast* gene. The animals were sacrificed with an overdose of pentobarbital sodium (100 mg/kg) after 12 h, and fluid accumulation was measured.

Colonization of the small intestine by *A. hydrophila* wild type and enterotoxin gene-deficient mutants. Mice challenged with various bacteria in the lumen of the small intestinal loops were sacrificed at 2 and 12 to 16 h. The small intestinal segments of the animals were washed with PBS to remove unbound bacteria and homogenized in PBS. Various dilutions were plated on MacConkey agar plates with 10 μ g of ampicillin (Remel, Lenexa, Kans.)/ml to selectively grow *A. hydrophila*. In some experiments, the tissues were homogenized in PBS and then mixed with the accumulated fluid after 12 to 16 h to determine the increase in the number of *A. hydrophila* organisms.

Statistical analysis. Wherever appropriate, the data were analyzed with the multiple-group comparison Tukey test, and *P* values of ≤ 0.05 were considered significant.

Nucleotide sequence accession number. The sequence of the *ast* gene has been submitted to GenBank with accession no. AF419157.

RESULTS

Expression of the *ast* gene from a 4.8-kb *Sal*I/*Bam*HI DNA fragment of the *A. hydrophila* SSU chromosome and DNA sequence analysis of the *ast* gene. Both Act and Alt enterotoxins have previously been molecularly characterized in our laboratory (15, 16, 17, 20, 21, 22). However, although the gene encoding Ast has been cloned, we only recently sequenced the gene (accession no. AF419157). The sequences of these enterotoxin genes, as well as those of their flanking DNA, were needed to prepare isogenic mutants of *A. hydrophila* deficient in production of one or more of these enterotoxins. This, in turn, allowed us to delineate their precise role in causing gastroenteritis.

To obtain an estimate of the potential length of the *ast* gene ORF and the direction of *ast* gene transcription, we expressed a 4.8-kb *Sal*I/*Bam*HI DNA fragment containing the *ast* gene in *E. coli* with a bacteriophage T7 expression system using vectors pT7-5 and pT7-6 (Table 1). This system provided us with two advantages: (i) the expression of the genes on the cloned DNA fragment could be exclusively monitored by [³⁵S]methionine labeling of the proteins, as *E. coli* protein synthesis was shut down by RIF, which did not affect T7 RNA polymerase activity, and (ii) pT7-5 and pT7-6 vectors had multiple cloning sites in opposite orientations, thereby allowing us to determine the direction of the *ast* gene transcription.

Our data indicated two radiolabeled polypeptides of 32 and 71 kDa when a 4.8-kb *Sal*I/*Bam*HI DNA fragment was expressed from vector pT7-6 in the direction of *Sal*I to *Bam*HI. No radioactive band was detected when we used a pT7-5 vector to insert this fragment in the opposite orientation (data not

shown). Our DNA sequence analysis of the 4.8-kb *SalI/BamHI* DNA fragment revealed this fragment to be 4,623 bp with three ORFs in the direction of *SalI* to *BamHI* at nucleotide positions 10 to 894 (encoding a 295-amino-acid-long polypeptide), 1007 to 1861 (encoding a 284-amino-acid-long polypeptide), and 1964 to 3874 (encoding a 636-amino-acid-long polypeptide) with approximate molecular sizes of 32, 31, and 71 kDa, respectively.

The cell lysate from this *E. coli* clone promoted CHO cell elongation (titer, 1:256) and caused fluid secretion (2.8 ± 0.2 ml/5 cm) in the rat ligated small intestinal loops. The cell lysate from an *E. coli* clone with vector alone or with the 4.6-kb *SalI/BamHI* DNA fragment inserted in the opposite orientation in vector pT7-5 did not cause any CHO cell elongation or fluid secretion and served as a negative control. Cholera toxin (1 μ g) and PBS were used as positive and negative controls, respectively, for the CHO cell and loop assay. The fluid secretion caused by cholera toxin was 3.2 ± 0.3 ml/5 cm of the loop. A total of 10 rats were used, each with an appropriate negative and positive control and the test samples. These data suggested Ast to be either 31 to 32 or 71 kDa in size.

We subsequently cloned a 3.6-kb *BamHI* fragment from the chromosomal DNA of another clinical isolate of *A. sobria*, which, when expressed with the T7 expression system (using the pT7-6 vector) in the correct orientation, exhibited enterotoxic activity similar to that seen with the 4.6-kb *SalI/BamHI* fragment from *A. hydrophila* SSU on CHO cells, as well as in rat ligated ileal loops. No biological activity was detected in those clones in which the 3.6-kb DNA fragment was inserted in the opposite orientation. The DNA sequence analysis of this 3.6-kb fragment revealed only one ORF at nucleotide positions 943 to 2853, which encoded a 636-amino-acid-long polypeptide similar to Ast from *A. hydrophila* SSU. A 91% homology was noted between Ast from these two isolates of *Aeromonas* at the amino acid level. These data indicated Ast to be 71 kDa in size. The DNA sequence (943 bp) upstream of the 5' end of the *ast* gene showed only 5% homology between 4.6-kb *SalI/BamHI* and 3.6-kb *BamHI* DNA fragments, while the homology was 87% in the region spanning 749 bp downstream of the 3' end of the *ast* gene. These data indicated a significant divergence in sequences flanking the *ast* gene in different species of *Aeromonas*. The DNA sequence of the *ast* gene, along with its deduced amino acid sequence, is available in GenBank (accession no. AF419157).

The highly purified Ast exhibited a size of 71 kDa after SDS-PAGE, and the NH₂-terminal sequence (5 amino acid residues sequenced) of the purified Ast matched the DNA-derived amino acid sequence. The availability of the sequence of the entire 4.6-kb *SalI/BamHI* fragment allowed us to develop an *ast* isogenic mutant of *A. hydrophila* to define its role in causing gastroenteritis.

Characterization of SSU Δ *alt* and SSU Δ *ast* mutants of *A. hydrophila* SSU. The strategies used to develop *alt* and *ast* isogenic mutants are depicted in Fig. 1. Conjugation of wild-type *A. hydrophila* SSU with *E. coli* S17-1 harboring either pJQ *alt*-Km or pJQ *ast*-Km with a truncated *alt* or *ast* gene, respectively, should have resulted in transconjugants that were resistant to RIF, KAN, and 5% sucrose but sensitive to GEN. Such mutants should have undergone genuine double-crossover homologous recombination, resulting in the replacement of na-

tive *alt* and *ast* genes with truncated *alt* and *ast* genes and concomitant loss of the suicide vector with Gm and *sacB* genes.

To confirm the identify of these isogenic mutants, the chromosomal DNA was isolated and subjected to PCR and Southern blot analysis. For PCR analysis, specific primers generated to either the *alt* and *ast* genes or the Km gene cassette were used (Table 2). These primers detected 1.1- and 1.9-kb DNA fragments from the chromosome of wild-type *A. hydrophila*, which represented the correct sizes of the *alt* and *ast* genes, respectively (20). However, the size of the *alt* and *ast* genes was larger by 1.2 kb in the isogenic mutants, due to the insertion of the Km gene cassette. Similarly, the primers to the Km gene cassette detected a 1.2-kb DNA fragment from the isogenic mutants but not from the chromosomal DNA of wild-type *A. hydrophila* (data not shown). The PCR data then were confirmed by performing Southern blot analysis.

As depicted in Fig. 2A, the size of the chromosomal DNA fragment from wild-type *A. hydrophila* digested with *SalI* restriction enzyme was 4.0 kb when an *alt*-specific gene probe was used (Fig. 2A-I, lane 2). However, the size of the *SalI* DNA fragment from the mutant SSU Δ *alt* was 5.2 kb due to the insertion of a Km gene cassette (Fig. 2A-I, lane 1). A similarly sized DNA fragment was detected in the digested chromosomal DNA of the mutant strain when the Km gene cassette was used as a probe (Fig. 2A-II, lane 1). This probe did not react with the digested DNA from the wild-type *A. hydrophila* (Fig. 2A-II, lane 2). No band was detected in the digested chromosomal DNA of both the mutant (Fig. 2A-III, lane 1) and wild-type *A. hydrophila* (Fig. 2A-III, lane 2), when the suicide vector pJQ200SK was used as a probe. The probe reacted with the pJQ200SK plasmid digested with *XbaI* restriction enzyme and served as a positive control (Fig. 2A-III, lane 3). These data indicated that the mutant strain SSU Δ *alt* had completely lost the suicide vector sequence as a result of double-crossover homologous recombination.

Similarly, we digested chromosomal DNA from mutant SSU Δ *ast* and wild-type *A. hydrophila* with restriction enzymes *SalI/BamHI* and subjected them to Southern blot analysis (Fig. 2B). As shown in Fig. 2B-I, lane 2, the digested chromosomal DNA from wild-type *A. hydrophila* exhibited a band of 4.6 kb when an *ast* gene-specific probe was used. The size of this fragment from the mutant strain SSU Δ *ast* was 5.8 kb due to the insertion of a Km gene cassette (Fig. 2B-I, lane 1). Although the 5.8-kb DNA fragment from the SSU Δ *ast* mutant reacted with the Km cassette gene probe (Fig. 2B-II, lane 1), it failed to react with chromosomal DNA from the wild-type *A. hydrophila* as expected (Fig. 2B-II, lane 2). Neither digested chromosomal DNA from the wild-type strain nor that from the mutant strain of *A. hydrophila* reacted with the suicide vector probe, as shown in Fig. 2B-III (lanes 1 and 2). Lane 3 in Fig. 2B-III represented a positive control showing the suicide vector probe hybridizing to itself.

Characterization of SSU Δ *alt,ast*, SSU Δ *act,ast*, and SSU Δ *act,alt* double-knockout mutants and an SSU Δ *act,alt,ast* triple-knockout mutant of *A. hydrophila*. Since we used the same plasmid, pDMS *ast*-Sm/Sp, for the generation of mutants SSU Δ *alt,ast* and SSU Δ *act,ast* (Fig. 1), the Southern blot patterns for these two mutants were similar, and therefore, a representative blot for only one of the mutants is shown here. The chromosomal DNA from these isogenic mutants and wild-type *A. hydrophila*

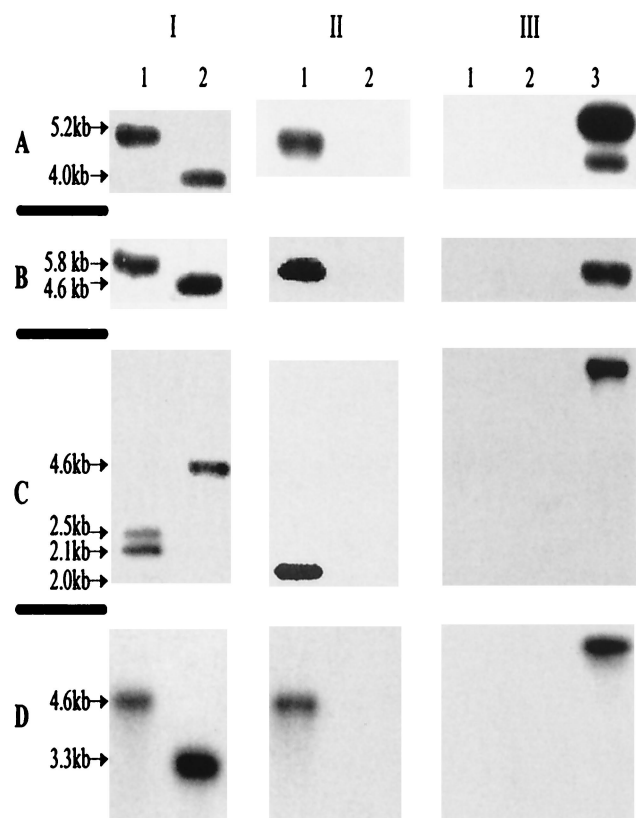


FIG. 2. Confirmation of the identity of the enterotoxin gene-deficient mutants of *A. hydrophila* SSU based on Southern blot analysis. (A) Chromosomal DNA from SSU Δ *alt* mutant (lanes 1) and wild-type *A. hydrophila* (lanes 2) was digested with *Sal*I restriction enzyme. Suicide vector pJQ200SK digested with restriction enzyme *Xba*I was used in lane 3. (B) Chromosomal DNA from the SSU Δ *ast* mutant (lanes 1) and wild-type *A. hydrophila* (lanes 2) was digested with *Sal*I/*Bam*HI restriction enzyme. Suicide vector pJQ200SK digested with restriction enzyme *Xba*I was used in lane 3. (C) Chromosomal DNA from the SSU Δ *alt,ast* or SSU Δ *act,ast* mutant (lanes 1) and wild-type *A. hydrophila* (lanes 2) was digested with *Sal*I/*Bam*HI restriction enzyme. Suicide vector pDMS197 digested with restriction enzyme *Xba*I was used in lane 3. (D) Chromosomal DNA from the SSU Δ *act,alt* or SSU Δ *act,alt,ast* mutant (lanes 1) and wild-type *A. hydrophila* (lanes 2) was digested with *Kpn*I/*Xba*I restriction enzyme. Suicide vector pRE112 digested with restriction enzyme *Xba*I was used in lane 3. Different enterotoxin genes (*alt* [A-I and D-I] and *ast* [B-I and C-I]), different antibiotic gene cassettes (Km cassette [A-II and B-II], Sm-Sp cassette [C-II], and Tc cassette [D-II]), and different suicide vectors (pJQ200SK [A-III and B-III], pDMS197 [C-III], and pRE112 [D-III]) were used as probes. In panel A, lane 3, the two bands were due to incomplete digestion of the vector pJQ200SK.

was digested with restriction enzymes *Sal*I/*Bam*HI and hybridized with various probes. In Fig. 2C-I, the *ast*-specific gene probe was used. Two bands of 2.1 and 2.5 kb were detected in the mutant strain (lane 1), whereas one 4.6-kb DNA fragment was detected in the wild-type *A. hydrophila* (Fig. 2C-I, lane 2). The detection of two bands in the mutant strain was expected, as the Sm-Sp gene cassette was flanked by a *Bam*HI site. Therefore, digestion of the chromosomal DNA from the mutant strain with *Sal*I/*Bam*HI enzymes removed the Sm-Sp gene cassette and divided the *ast* gene into two DNA fragments of 2.1 and 2.5 kb. In Fig. 2C-II, lane 1, a 2.0-kb band representing the antibiotic gene cassette was detected in the mutant but not

in the wild-type *A. hydrophila* (lane 2) when a Sm-Sp gene cassette was used as a probe. In Fig. 2C-III, lanes 1 and 2, the chromosomal DNA from both the mutant and the wild-type *A. hydrophila* did not react with the pDMS197 vector probe, indicating loss of the suicide vector from the chromosome of the mutant.

Because the same plasmid, pRE *alt*-Tc, was used for generation of mutants SSU Δ *act,alt* and SSU Δ *act,alt,ast* (Fig. 1), the Southern blot patterns for these two mutants were similar, and therefore, a representative blot for only one of the mutants is shown here. The chromosomal DNA from these two mutant strains as well as that from the wild-type *A. hydrophila* was digested with restriction enzymes *Kpn*I/*Xba*I. In Fig. 2D-I, the *alt* gene was used as a probe, which detected a 4.6-kb DNA fragment in the mutant strain (lane 1). This fragment was larger by 1.3 kb than the one seen in wild-type *A. hydrophila* (Fig. 2D-I, lanes 1 and 2) due to the insertion of the Tc gene cassette. The size of the DNA fragment detected in the wild-type *A. hydrophila* was 3.3 kb (Fig. 2D-I, lane 2). In Fig. 2D-II, lane 1, a similarly sized DNA fragment (4.6 kb) was detected in the mutant strain when a Tc gene cassette was used as a probe, whereas this probe failed to react with the wild-type *A. hydrophila* chromosomal DNA (Fig. 2D-II, lane 2). As expected, neither the chromosomal DNA from the mutant nor that from the wild-type *A. hydrophila* reacted with the suicide vector probe (Fig. 2D-III, lanes 1 and 2). The Southern blot data obtained with the double- and triple-knockout mutants were confirmed by PCR analysis using toxin gene-specific primers and by DNA sequence analysis of the PCR product using primers to the toxin genes (Table 2). The DNA sequence analysis revealed insertion of antibiotic resistance genes at the correct location in these mutants (data not shown).

Northern blot analysis to demonstrate loss of transcription of the corresponding enterotoxin genes in the isogenic mutants of *A. hydrophila* SSU. Total RNA from various mutants and wild-type *A. hydrophila* was isolated and subjected to Northern blot analysis by using *act*-, *alt*-, and *ast*-specific gene probes. As shown in Fig. 3A, an *act* gene transcript of 1.4 kb was detected in the wild-type *A. hydrophila* (lane 1) and in mutants SSU Δ *alt* (lane 3), SSU Δ *ast* (lane 4), and SSU Δ *alt,ast* (lane 5), in which the *act* gene was intact. A similar level of hemolytic activity was noted in the culture filtrates of these mutants (data not shown). However, in mutants SSU Δ *act* (lane 2), SSU Δ *act,alt* (lane 6), SSU Δ *act,ast* (lane 7), and SSU Δ *act,alt,ast* (lane 8), no *act* gene transcript was detected, indicating the successful truncation of the *act* gene in these mutants. Such mutants also did not exhibit any hemolytic activity.

In Fig. 3B, we used an *alt*-specific gene probe, and a 1.9-kb transcript was detected in wild-type *A. hydrophila* (lane 1) and in mutants SSU Δ *act* (lane 2), SSU Δ *ast* (lane 4), and SSU Δ *act,ast* (lane 7). No *alt*-specific transcript was detected in mutants SSU Δ *alt* (lane 3), SSU Δ *alt,ast* (lane 5), SSU Δ *act,alt* (lane 6), and SSU Δ *act,alt,ast* (lane 8). Surprisingly, no *ast* gene transcription was detected in wild-type *A. hydrophila* and its various enterotoxin gene-deficient mutants (data not shown). These data possibly suggested (i) a very low level of the transcript for the *ast* gene, (ii) a short half-life of the *ast* mRNA, and (iii) rapid degradation of the *ast* gene transcript.

Western blot analysis to demonstrate the presence of Ast in

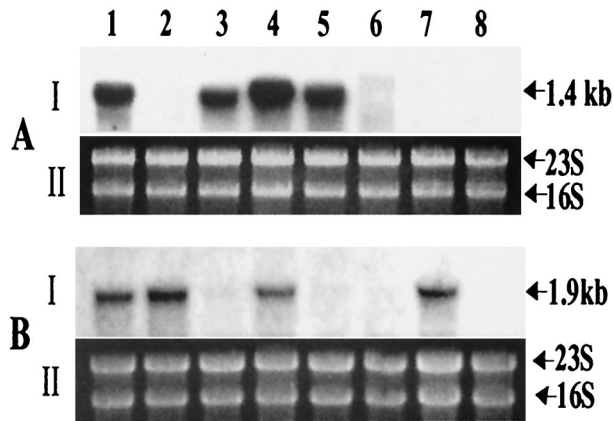


FIG. 3. The transcripts for the enterotoxin genes *act* and *alt* were eliminated in the indicated mutants, based on Northern blot analysis. Total RNA from wild-type *A. hydrophila* and its enterotoxin gene-deficient mutants was isolated and hybridized with the *act* gene probe (A-I) and the *alt* gene probe (B-I), as described in Materials and Methods. Lane 1, wild-type *A. hydrophila*; lane 2, mutant SSU Δ *act*; lane 3, mutant SSU Δ *alt*; lane 4, mutant SSU Δ *ast*; lane 5, mutant SSU Δ *alt,ast*; lane 6, mutant SSU Δ *act,alt*; lane 7, mutant SSU Δ *act,ast*; lane 8, mutant SSU Δ *act,alt,ast*. The RNA loaded in each lane was quantitated by scanning 16S and 23S rRNA bands after ethidium bromide staining of the gel (A-II and B-II).

wild-type *A. hydrophila* SSU and its isogenic mutants. Data presented in Fig. 4 clearly show the presence of Ast antigen in wild-type *A. hydrophila* (lane 1) and all of the isogenic mutants that had an intact *ast* gene (lanes 2, 3, and 6) in Western blots probed with Ast-specific antibodies. The Ast-specific band was missing from the isogenic mutants SSU Δ *ast* (lane 4), SSU Δ *alt,ast* (lane 5), SSU Δ *act,ast* (lane 7), and SSU Δ *act,alt,ast* (lane 8). Purified Ast was used as a positive control (lane 9), and cell lysates from *E. coli* served as a negative control (lane 10) to demonstrate the specificity of Ast antibodies.

Evaluation of various isogenic mutants of *A. hydrophila* in a mouse model. A diet-restricted, antibiotic-treated adult mouse model was used to evaluate different isogenic mutants of *A. hydrophila* for enterotoxin activity. This model provided optimal fluid secretion after *A. hydrophila* challenge. In animals fed a normal diet and challenged with wild-type *A. hydrophila* (5×10^5 CFU), a mean fluid secretion of 175 ± 55 μ l/5 cm of the

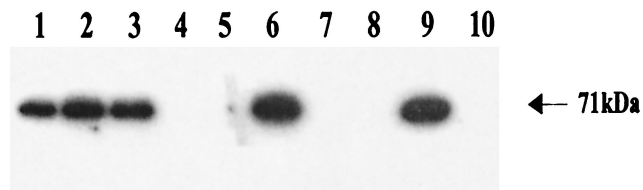


FIG. 4. Western blot analysis showing that the *ast* gene expression was eliminated in its corresponding gene-deficient mutant of *A. hydrophila*. The cell lysates from different enterotoxin-deficient mutants of *A. hydrophila* were subjected to SDS-12% PAGE, and subsequently the proteins were transferred to a nitrocellulose membrane for Western blot analysis as described in Materials and Methods. Lane 1, wild-type *A. hydrophila*; lane 2, SSU Δ *act*; lane 3, SSU Δ *alt*; lane 4, SSU Δ *ast*; lane 5, SSU Δ *alt,ast*; lane 6, SSU Δ *act,alt*; lane 7, SSU Δ *act,ast*; lane 8, SSU Δ *act,alt,ast*; lane 9, purified Ast (0.1 μ g) as a positive control; lane 10, cell lysate from *E. coli* as a negative control.

TABLE 3. Ability of wild-type *A. hydrophila* SSU, single-knockout mutants, and their complemented strains to cause fluid secretion in a mouse model

Organism injected (2×10^5 /100 μ l) ^a	No. of BALB/c mice tested	Fluid accumulation (μ l/5-cm loop \pm SD)	% Reduction in fluid secretion
WT <i>A. hydrophila</i>	25	850 ± 50^c	0
SSU Δ <i>act</i>	25	$300 \pm 29^{b,c}$	64
SSU Δ <i>alt</i>	25	$445 \pm 28^{b,c,d}$	48
SSU Δ <i>ast</i>	25	$485 \pm 22^{b,c,d}$	43
WT <i>A. hydrophila</i> (pBR322)	25	812 ± 44	0
SSU Δ <i>alt</i> (pBalt)	25	820 ± 24	0
SSU Δ <i>ast</i> (pBast)	25	805 ± 20	0
SSU Δ <i>act</i> (pBact)	25	868 ± 25	0

^a The number of organisms in each loop after 12 to 16 h was increased by 2 logs. WT, wild type.

^b All groups were significantly different from wild-type *A. hydrophila* at $P < 0.001$ by multiple-group comparison Tukey test.

^c All groups were significantly different from each other at $P < 0.001$ by multiple-group comparison Tukey test.

^d These groups were not statistically different from each other ($P = 0.056$).

loop was recorded over a 12- to 16-h observation period. The fluid accumulation was 912 ± 28 μ l/5 cm in animals which were food restricted but not treated with STR. In food-restricted and STR-treated mice, the fluid accumulation increased to $1,378 \pm 38$ μ l/5 cm. A total of 10 mice in each group were used in the above-mentioned study. We noted that, with the SSU Δ *alt,ast* mutant, the fluid secretion occurred rapidly, with maximum fluid levels noted in 4 h. This mutant induced fluid secretion as a result of Act production, as the *alt* and *ast* genes were deleted from this strain. However, the fluid secretion evoked by Alt and Ast in an *act* isogenic mutant of *A. hydrophila* was minimal within 4 to 6 h, becoming maximal only after 12 h. Therefore, we opted to observe fluid secretion by various enterotoxin gene-deficient mutants of *A. hydrophila* after 12 to 16 h of observation.

As shown in Table 3, single-knockout mutants, such as SSU Δ *act*, SSU Δ *alt*, and SSU Δ *ast*, reduced fluid secretion by 64, 48, and 43%, respectively, compared to fluid secretion evoked by the wild-type *A. hydrophila*. The enterotoxin activity of mutants SSU Δ *alt*, SSU Δ *ast*, and SSU Δ *act* was restored after complementation. The wild-type *A. hydrophila* with pBR322 vector alone was used as a control in the complementation experiments. The differences in fluid accumulation evoked by various mutants not only were significant compared to the wild-type bacterium but also exhibited significance when these mutants were compared to one another by using Tukey multiple-group comparison analysis (Table 3). The only mutants which demonstrated no statistically significant difference in fluid accumulation were SSU Δ *alt* and SSU Δ *ast*, which culminated in 445 ± 28 and 485 ± 22 μ l of fluid/5 cm of the loop, respectively. It was noted that, compared to fluid secretion of $1,378 \pm 38$ μ l/5 cm of the loop observed in a different experiment (as stated above) using diet-restricted, antibiotic-treated animals, the amount of fluid accumulated was less in the experiments presented in Tables 3 and 4, indicating variation in animals purchased at different times.

In the SSU Δ *alt,ast* mutant, the combined reduction in fluid secretion due to Alt and Ast was 36%, and Act accounted for 64% of the total fluid accumulated (Table 4). For the SSU Δ *act,ast* mutant, which would cause fluid secretion only

TABLE 4. Ability of wild-type *A. hydrophila* SSU, double-knockout mutants (SSU Δ alt,ast, SSU Δ act,ast, and SSU Δ act,alt), and triple-knockout mutant (SSU Δ act,alt,ast) to cause fluid secretion in a mouse model

Organism injected ($2 \times 10^5/100 \mu\text{l}$) ^a	No. of BALB/c mice tested	Fluid accumulation ($\mu\text{l}/5\text{-cm}$ loop \pm SD)	% Reduction in fluid secretion
WT <i>A. hydrophila</i>	25	858 \pm 28 ^b	0
SSU Δ alt,ast	25	552 \pm 26 ^b	36
SSU Δ act,ast	25	330 \pm 28 ^b	62
SSU Δ act,alt	25	230 \pm 18 ^b	73
SSU Δ act,alt,ast	25	ND ^c	100

^a The number of organisms in each loop after 12 to 16 h was increased by 2 logs. WT, wild type.

^b All groups were significantly different from each other at $P < 0.001$ by multiple-group comparison Tukey test.

^c ND, not detected. The limit of detection was less than 50 μl .

through Alt production, 38% of the enterotoxigenic activity was noted, compared to that observed for the wild-type *A. hydrophila*. In a double-knockout mutant in which *act* and *alt* genes were deleted, fluid secretion totaling $230 \pm 18 \mu\text{l}/5 \text{ cm}$ of the loop was measured, indicating the contribution of Ast to be 27% (Table 4). No detectable fluid secretion was noted in the mutant strain in which all three enterotoxin genes were deleted. Once again, the differences in fluid accumulation in these various mutants were statistically significant compared either to the wild-type bacterium or among one another with the Tukey test. All of these mutants colonized the small intestine of mice to the same extent [$(1.0 \pm 0.6) \times 10^5$ to $(1.2 \pm 0.6) \times 10^5$ CFU/ml] as that noted for the wild-type *A. hydrophila* ($1.1 \times 10^5 \pm 0.8 \times 10^5$ CFU/ml) after 2 h, and their number increased by approximately 2 logs after 12 to 16 h of incubation.

DISCUSSION

We have performed, for the first time, an extensive case-control human study at the International Center for Diarrheal Diseases Research, Dhaka, Bangladesh, which included children younger than 5 years of age, to demonstrate the extent of *Aeromonas* infection in these children. We indeed established an association of *Aeromonas* with diarrhea and showed that enterotoxins were involved in *Aeromonas*-associated gastroenteritis (3). Our recent studies also indicated that bloody diarrhea was most commonly associated with the production of the cytotoxic enterotoxin Act, while nonbloody diarrhea was correlated with the elaboration of the cytotoxic enterotoxins Alt and Ast (3; unpublished data). Diarrheal isolates harboring one, two, or all three of the enterotoxin genes were recovered from patients, although most of the *Aeromonas* isolates contained two of the three enterotoxin genes in various combinations. These studies were, therefore, designed to delineate precisely the roles of individual enterotoxins in evoking diarrhea by developing mutants with various combinations of deletions of enterotoxin genes.

Although the genes encoding Act and Alt were previously sequenced in our laboratory (17, 20), the DNA sequence of the *ast* gene was not available and was needed to generate an *ast* isogenic mutant. Based on the expression of a 4.6-kb *SalI*/*Bam*HI DNA fragment in *E. coli* with the T7 expression sys-

tem, biological activity measurement and subsequent DNA sequence analysis revealed that Ast was 71 kDa in size. The size of Ast was further confirmed by its molecular characterization from another clinical isolate of *A. sobria* and by the development of an *ast* isogenic mutant. The SSU Δ ast mutant truncated within the *ast* gene indeed exhibited a reduced enterotoxigenic activity, and biological activity associated with Ast was restored after complementation (Table 3).

Our initial strategy was to use purified enterotoxins individually or in various combinations to demonstrate their role in evoking fluid secretion and to determine any synergism among these enterotoxins. However, we noted that, when the *ast* gene was hyperexpressed with the T7 polymerase-promoter-based pT30a vector system, most of the Ast was membrane bound, requiring harsh treatment for solubilization (e.g., 8 M urea), thereby resulting in the loss of biological activity. We encountered a similar solubility problem when *act* and *alt* genes were hyperexpressed in *E. coli* (20, 25). However, we circumvented the Act and Alt solubility problem by expressing the corresponding genes in *A. salmonicida* and *A. hydrophila* strains, lacking *act* and *alt* genes, respectively, by using a broad-host-range pMMB66 vector with an IPTG-inducible *tac* promoter (22; unpublished data). This vector system allowed Act and Alt proteins to be secreted out into the medium in a biologically active form (22; unpublished data). The *act* and *alt* gene-negative strains of *Aeromonas* were needed to prevent homologous recombination between the toxin gene on the recombinant pMMB66 plasmid and on the chromosome.

We were unable to express the *ast* gene by using this vector, because until recently we did not have an *Aeromonas* mutant with a complete deletion of the *ast* gene to prevent homologous recombination between the toxin gene on the recombinant pMMB66 plasmid and that on the chromosome. These potent enterotoxins are produced in small amounts from the bacterium, and hyperexpression of these toxin genes is essential for generating meaningful data on their relative contributions in evoking fluid secretion. We therefore opted to prepare isogenic mutants deficient in various enterotoxin genes to define their role in causing diarrhea in a murine model.

Inactivation of the coding region of genes by insertion of antibiotic resistance markers is a general strategy for the construction of defined mutants (9, 24, 36, 44). The use of suicide vectors is fundamental to this technique, allowing the creation of deletions or insertions in specific genes on chromosomes (24). However, once the suicide vector is integrated into the chromosome, it is necessary to remove the vector DNA, resulting in replacement of the wild-type allele with a mutant allele. A suicide vector carrying a conditional lethal gene, such as a *sacB* gene, that discriminates between the integration of the vector and double-recombination events has been widely used (45). Therefore, all of the suicide vectors used in this study (pJQ200SK, pDMS197, and pRE112) (Fig. 1 and Table 1) harbored the *sacB* gene. The *sacB* gene, which encodes levansucrase, probably polymerizes levan, a product of catabolism of sucrose in the periplasm of gram-negative bacteria, which is toxic to bacteria when they are grown in the presence of sucrose (23). Vectors pDMS197 and pRE112 had a conditional R6K *ori* and required π protein for replication, while pJQ200SK had a P15A *ori* from plasmid pACYC184 (24, 28).

In this study, six isogenic mutants of *A. hydrophila*, in which three enterotoxin genes were deleted in various combinations, were prepared. The biological activity of the single-knockout strains (SSU Δ act, SSU Δ alt, and SSU Δ ast) of *A. hydrophila* could be restored by complementation, suggesting no polar effects of marker-exchange mutagenesis on genes downstream of those encoding the enterotoxins. Since double- and triple-enterotoxin-gene-knockout strains of *A. hydrophila* were prepared using one of the single-enterotoxin-gene-knockout strains, we did not expect polar effects. However, complementation of double- and triple-enterotoxin-gene-knockout strains of *A. hydrophila* was difficult to perform to completely rule out the possibility of any polar effects in such mutants. We determined the exact insertional sites of the antibiotic resistance cassettes within the enterotoxin genes on the chromosomes of various isogenic mutants and confirmed the absence of frameshift mutations within the toxin genes. All of these mutants (single, double, and triple knockout) exhibited similar growth rates and the ability to colonize and multiply in the small intestine, which further suggested the absence of polar effects in these mutants. However, we should not overlook the possibility of polar effects in the double- and triple-knockout strains of *A. hydrophila*, which could have some impact on the fluid accumulation results. Such polar effects might constitute a limitation of insertional mutagenesis.

We noted previously and during present studies that the frequency of double crossover was very low (0.01%) when the mutants SSU Δ act, SSU Δ alt, and SSU Δ ast were prepared using suicide vector pJQ200SK (47). We therefore employed recently developed suicide vectors pDMS197 and pRE112 (24) for generating double- and triple-knockout mutants of *A. hydrophila*, which increased the frequency of obtaining double-crossover mutants by 10-fold. Further, it was noted that, when the pJQ200SK suicide vector was used, some false-positive mutants were observed, especially during the preparation of double- and triple-knockout strains of *A. hydrophila* that were truncated in two and three enterotoxin genes, respectively. The majority of the false-positive mutants still had a portion of the suicide vector plasmid integrated in the chromosomal DNA of *A. hydrophila*, based on Southern blot data (data not shown). These mutants, however, exhibited the correct phenotype (i.e., resistance to sucrose and sensitivity to GEN). Our subsequent analysis of these mutants indicated that Gm^r and *sacB* genes were indeed lost from these cultures (data not shown). In some instances, we could also detect free recombinant pJQ200SK plasmid, suggesting its replication to some extent in *A. hydrophila*, which could also interfere with obtaining genuine double-crossover mutants. The frequency of obtaining false-positive mutants was significantly reduced with pDMS197 and pRE112 vectors.

The correct identity of all of the isogenic mutants was confirmed by Southern, Northern, and Western blot analysis (Fig. 2 to 4). In Northern blot analysis, all of the mutants exhibited the correct pattern of inhibition of the corresponding enterotoxin gene transcripts when *act* and *alt* gene probes were used (Fig. 3). However, it was noted that the *alt* mRNA level was significantly lower than that of *act* mRNA (Fig. 3A and B). These data suggested (i) low levels of the *alt* gene expression, under studied conditions, and (ii) instability or shorter half-life of the *alt* mRNA. It will be intriguing to determine in the

future whether *alt* gene expression could be increased during in vivo conditions. Interestingly, we were unable to detect a transcript for the *ast* gene, even in the wild-type *A. hydrophila*, although the Ast protein could be seen by Western blot analysis in wild-type *A. hydrophila* and its isogenic mutants with an intact *ast* gene (Fig. 4). Our data might be suggestive of a short half-life for the *ast* mRNA or its rapid degradation and need confirmation. This possibility was based on our observation that we could detect the *ast* gene transcript in significant amounts from *E. coli* when the toxin gene was hyperexpressed using a pET30a vector system (data not shown). However, we cannot rule out the possibility that the *ast* gene expression requires interaction of *A. hydrophila* with the host cell, and this is under investigation.

We first developed a mouse model to evaluate the fluid secretory potential of wild-type *A. hydrophila* and its various enterotoxin gene-deficient mutants, based on studies showing that malnourished animals were more susceptible to infection than were adequately nourished animals (37). Further, STR disrupted the aerobic and anaerobic characteristics of the cecal flora and was particularly effective at decreasing colonization resistance (43). Deletion of the enterotoxin genes individually indicated that Act, Alt, and Ast contributed to the fluid secretory response by 64, 48, and 43%, respectively (Table 3). Data obtained with SSU Δ act,ast and SSU Δ act,alt mutants, which caused fluid secretion via the production of Alt and of Ast, respectively, conclusively proved their contribution to be 38 and 27%, respectively (Table 4). These data coincided with our observation that mice immunized with Alt and Ast reduced fluid secretion caused by *A. hydrophila* challenge by 39 and 30%, respectively (20; unpublished data).

Based on the data presented in Tables 3 and 4 for SSU Δ act and SSU Δ alt,ast mutants, it was evident that Act was the major enterotoxin contributing to the fluid secretory response (64%), followed by Alt and Ast. Our data also suggested some interaction among these various enterotoxins (Tables 3 and 4). The exact molecular basis of this interaction among the various enterotoxins is not known yet and needs further study. The inability of the triple-knockout mutant to exhibit any detectable enterotoxic response confirmed the role of all three of the enterotoxins (Act, Alt, and Ast) in *A. hydrophila*-induced diarrhea. The presence of various combinations of enterotoxins in different *Aeromonas* isolates could increase or decrease the expression of specific enterotoxin genes and thus could dictate the severity of diarrhea. The availability of purified enterotoxins would help in further delineating interactions among these enterotoxins.

In conclusion, we have demonstrated for the first time the contribution of each of the three enterotoxins of *A. hydrophila* in causing gastroenteritis in a murine model by developing isogenic mutants. Our data tend to suggest some interaction among these enterotoxins in vivo that could lead to altered fluid secretion. Such attenuated strains of *A. hydrophila* with deletions of the *act*, *alt*, and *ast* genes could be attractive candidates for vaccine development.

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Jian Sha and E. V. Koslova contributed equally to this work.

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